Rajoli Keerthi et al, 2026, Vol.14, No.4: 85-92

International Journal of Chemistry and Pharmaceutical Sciences

https://doi.org/10.30904/j.ijcps.2026.5013 | E-ISSN: 2321-3132

In-Vitro and In-Vivo Assessment of Selected Contraceptive Potential

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Received: 01-02-2026 | Revised: 05-03-2026 | Accepted: 10-04 2026 | Published: 30-04-2026

Abstract:

The present study comprehensively evaluated the contraceptive potential of *Justicia gendarussa* extract through both in vitro and in vivo investigations. The extract was successfully prepared via hydro-alcoholic maceration and Soxhlet extraction, yielding a phytochemically rich product containing flavonoids, alkaloids, tannins, saponins, and steroids. Quality control, chromatographic fingerprinting, and stability studies confirmed the extract's integrity, reproducibility, and pharmaceutical suitability. In vitro assays demonstrated that the extract preserved sperm viability and motility at lower concentrations but induced a dose-dependent decline at higher doses (≥1000 µg/mL). CASA analysis revealed significant reductions in motility and velocity parameters only at supra-physiological levels. Acrosome reaction studies confirmed intact responsiveness up to 500 µg/mL, with partial inhibition observed at higher doses. Enzyme inhibition assays highlighted selective suppression of hyaluronidase and acrosin at higher concentrations, suggesting potential mechanistic targets. Cytotoxicity and oxidative stress markers indicated minimal toxicity, while ELISA confirmed no disruption of systemic reproductive hormone balance. In vivo studies in Wistar rats validated these findings. Acute oral toxicity testing revealed an LD_{50} >2000 mg/kg, confirming a wide safety margin. Estrous cycle monitoring showed no disruption at baseline. Anti-implantation studies demonstrated significant dose-dependent inhibition (26–76%), with effects at higher doses comparable to levonorgestrel. Pregnancy outcome studies confirmed reduced fertility indices, smaller litter sizes, and higher resorption rates in treated groups. Importantly, reversibility was established, as normal fertility and litter sizes were restored after a 30-day washout period. Thus, *Justicia gendarussa* emerges as a promising candidate for non-hormonal, reversible contraception, aligning with the global need for safer, plant-based alternatives.

Keywords: *Justicia gendarussa*, Soxhlet extraction, Anti-implantation studies, Contraceptive Potential, CASA

Introduction

Test Substances (*Justicia gendarussa*)

Fig.1: *Justicia gendarussa*

Taxonomy & Nomenclature

- Family: Acanthaceae
- Accepted name: *Justicia gendarussa* Burm.f.
- Common synonyms: *Gendarussa vulgaris* Nees; *Adhatoda subserrata* Nees (others recorded in floras).

Vernacular Names

Gandarusa (Indonesia), Daun Rusa (Malay), Warer/Water Willow, Willow-leaved Justicia.

Distribution & Habitat

Native to tropical Asia (Indian subcontinent, Indochina, Malesia, New Guinea), naturalized or cultivated more widely; shrub of seasonally dry to moist lowland habitats.

Botanical Description

- Habit: Small, erect, much-branched evergreen shrub to ~1.5 m.
- Leaves: Opposite, lanceolate, 7–14 cm × 1–2.5 cm, entire, glabrous; apex acuminate.
- Inflorescence: Slender spikes to ~10 cm; flowers white with purple streaks/spots on the throat; subsessile.
- Fruit: Club-shaped, smooth 4-seeded capsule (~12 mm).

opposite narrow leaves; dark-purplish nodes; terminal/axillary pale flowers with purple striae; slender spike.

Ethnomedicinal

Widely used in South/Southeast Asian traditional medicine (e.g., jamu in Indonesia) for rheumatism, pain, respiratory ailments; leaves also used as insect-repellent.

Phytochemistry

- Flavonoids: Gendarusin A & B (apigenin C-glycosides; key markers in "gandarusa" leaf preparations); other apigenin/kaempferol glycosides.

- Other classes: Fatty acids, sterols (β-sitosterol), triterpenes; recently reported 2-aminobenzyl derivatives (justidrusamides A–D).

Organ/processing effects: Secondary-metabolite profiles vary by plant organ and culture conditions (important for standardizing leaf vs flower extracts).

Pharmacology

Reported antioxidant, anti-inflammatory/antinociceptive, antimicrobial, hepatoprotective, and other activities in preclinical studies and reviews. Patentiflorin A, an arylnaphthalene lignan from roots/stems, shows potent anti-HIV-1 activity in vitro against drug-resistant strains.

Evidence for Contraceptive Potential (male, non-hormonal)

- Traditional use: Male contraception reported among Papuan groups; developed locally as an herbal product candidate.
- Proposed mechanism: Reversible inhibition of sperm hyaluronidase, a key enzyme for cumulus/zona penetration; gendarusin A/B are leading actives.
- Human data (status): Small early clinical studies in Indonesia have been reported; scientific reviews note encouraging signals but emphasize that rigorous, published Phase-3 trials are still needed.

Quality Control & Standardization

- Marker compounds: Quantify gendarusin A (±B) by HPTLC/HPLC for lot-to-lot consistency; record full chromatographic fingerprints.
- Specimen integrity: Authenticate botanically (voucher), and control drying conditions that affect flavonoid content.

Materials and Methods

Cultivation & Propagation

Prefers sun to semi-shade; tolerates varied soils but grows best with moisture and drainage; propagated easily by stem cuttings—useful for generating consistent research material.

Collection, Authentication, and Processing

1. Collection & authentication: Collect the plant part (e.g., flowers/leaves/seeds) from a defined locality/season; authenticate by a qualified taxonomist; deposit voucher specimen (No 345/2025, department of botany, O.U).
2. Pre-processing: Wash with distilled water, shade-dry at 25–28 °C (RH 40–50%) to constant weight; mill; pass through 40-mesh sieve; store at 4 °C in amber, airtight containers with desiccant.

Extraction & Fractionation

- Hydro-alcoholic extract (70% methanol) by maceration or Soxhlet Ratio 1:10 (w/v); 48 h maceration with stirring (150 rpm) at room temperature or Soxhlet 6–8 h (≤40 °C bath). Filter (Whatman No.1). Concentrate under reduced pressure at ≤40 °C. Lyophilize aqueous residue.

Yield: %Yield = (Dry extract / Dry plant powder) × 100.

Standardization & Quality Control

- Preliminary phytochemistry: Alkaloids, flavonoids, tannins, saponins, steroids (qualitative tests).
- Total phenolics/flavonoids: Folin–Ciocalteu (mg GAE/g), $AlCl_3$ (mg QE/g).
- Chromatographic fingerprint: HPTLC/HPLC (define mobile phase, λmax). Record retention times, peak areas; system suitability (resolution, tailing, N).
- Residual solvents/microbial limits: As per pharmacopeial standards.
- Stability: 40 °C/75% RH, 30 days; re-assay potency (T0, T15, T30).

Test Item Preparation & Vehicles

- Stock for in vitro: Dissolve in DMSO or ethanol; final assay solvent ≤ 0.5% v/v (preferably ≤0.2%) after dilution with PBS or capacitation medium.
- Stock for in vivo: Suspend in 0.5% w/v CMC-Na (10 mL/kg p.o.). Prepare fresh daily.
- Concentration ranges: In vitro 1–1000 µg/mL (8-point half-log series). In vivo pilot 50, 100, 200 mg/kg/day, adjusted per acute toxicity.

Reagents and Kits for Biochemical/Functional Assays

- Media & buffers: PBS (pH 7.4), Tyrode's/HTF/BWW capacitation medium (37 °C, 5% CO_2), 0.5% BSA.
- Sperm assays:
  - CASA system (phase-contrast microscope, camera, software; 20 µm chamber).
  - Viability stains: Eosin–Nigrosin or Hoechst 33258.
  - Acrosome probes: FITC-PNA or FITC-PSA; calcium ionophore A23187 (positive AR inducer).
  - Spermicidal positive control: Nonoxynol-9 (low concentration benchmark).
- Enzyme kits: Hyaluronidase (turbidimetric or plate-based hyaluronan substrate); acrosin.
- Cytotoxicity & oxidative stress: MTT; LDH release; TBARS (MDA), GSH, SOD, CAT kits.
- ELISA (rat): FSH, LH, estradiol (E2), progesterone (P4), testosterone (T).
- Histology: 10% neutral buffered formalin (NBF); H&E, PAS; optional IHC (ERα/PR, AR, PCNA).

Laboratory Animals

- Species/strain: Wistar rats and/or Swiss albino mice.
- Age/weight: Rats—♀ 10–12 weeks (180–220 g), ♂ 12–14 weeks (220–260 g); Mice—♀ 8–10 weeks (20–25 g).
- Housing: Polypropylene cages (≤3 per cage), corn-cob bedding, 22 ± 2 °C, 12 h light/dark, RH 40–60%.
- Diet/water: Standard pellet diet; water ad libitum.
- Acclimatization: ≥7 days.
- Ethics: Prior IAEC approval; CPCSEA compliance.

In Vitro Studies

Sperm Motility and Viability Assay

Source: Fresh cauda-epididymal sperm from adult rats.

Collection: Dissect cauda epididymis; mince in pre-warmed (37 °C) BWW + 0.5% BSA; allow swim-out 10 min; filter through 80 µm mesh.

1. Standardization: Adjust to 20–40 × 10^6 sperm/mL; equilibrate 10 min at 37 °C, 5% CO_2.
2. Treatment: Aliquot 100–200 µL; add test item to final 1–1000 µg/mL (vehicle ≤0.5%); include vehicle control and nonoxynol-9 (positive control).

3. Incubation times:5,15,30 min (optional 60 min) at 37 °C.
4. CASA settings: Chamber depth 20 µm; frame rate ≥60 fps; ≥5 fields per well; record PR%, VCL, VSL, VAP, LIN, ALH, BCF. Maintain medium pH 7.2–7.4, osmolality 290–320 mOsm/kg.
5. Viability: Eosin–Nigrosin (mix 1:1; smear; count ≥200 cells) or Hoechst 33258 (fluorescence; 365 nm).
6. Data: Express as % progressive motility and % viable vs control; compute MEC (minimum effective concentration to reduce motility by 50% or 100% at specified timepoint).

Acceptance/Controls

- Intra-assay CV for PR% ≤10%.
- Vehicle should not alter PR% by >5% absolute.
- Temperature maintained at 37 ± 0.5 °C throughout.

Acrosome Reaction (AR) Test — Fluorescent Staining

Principle: Assess acrosome integrity/reaction using lectin probes (**FITC-PSA**).

Procedure

1. Capacitation: Incubate sperm 60 min in capacitation medium (BWW + BSA) at 37 °C, 5% CO_2.
2. Induction: Add A23187 (10 µM, 15 min) to induce AR (positive control).
3. Treatment: Pre-incubate with test item 15 min before AR induction or co-incubate during AR.
4. Staining: Wash; fix (2–4% paraformaldehyde, 10 min); incubate with FITC-PNA or FITC-PSA (10–20 µg/mL, 15 min, RT, dark). Counter-stain nuclei with DAPI/Hoechst if required.
5. Imaging/Counting: Fluorescence microscope; score ≥200 sperm/sample as acrosome-reacted (AR^+) or intact (AR^-).
6. Outcome/Calculation:
    - %AR (induced) in controls vs %AR (treated).
    - %AR inhibition = 100 × [1 − (AR%_treated / AR%_ induced control)].

Quality

- Positive (A23187) must raise AR% ≥2× basal.
- Exclude samples with baseline viability <70%.

Enzyme Inhibition Studies

(a) Hyaluronidase Inhibition

1. Prepare enzyme in assay buffer per kit (e.g., pH 5.0 acetate buffer).
2. Pre-incubate enzyme with test item (0–1000 µg/mL) for 10 min at 37 °C.
3. Add hyaluronan substrate; incubate 15–30 min; stop reaction.
4. Read turbidity/absorbance at λ specified by kit (commonly 600 nm).
5. Calculate % inhibition vs vehicle; derive IC_{50} by 4-parameter logistic fit.

(b) Acrosin Inhibition (BAPNA/Bz-Arg-pNA)

1. Pre-incubate acrosin with test item (0–1000 µg/mL), 10 min, 37 °C.
2. Add BAPNA substrate; incubate 10–20 min at 37 °C.
3. Measure p-nitroaniline release at 405 nm.
4. Compute enzyme activity (ΔA/min); % inhibition and IC_{50}.

Selectivity index (optional): SI = **CC_{50} (cytotoxicity on somatic cells) / IC_{50} (enzyme or motility)**.

In Vivo Studies

Experimental Animals

- Use healthy adult Wistar rats.
- Randomization: Computer-generated blocks; n = 8–10/sex/group (adjust by power).
- Blinding: Assessors for implantation counts, histology, ELISA blinded to allocation.

Acute Oral Toxicity (OECD 423, Up-and-Down)

1. Dosing sequence (females preferred): Start at 300 mg/kg; if no mortality, escalate to 2000 mg/kg with prescribed intervals.
2. Observation: Continuous for first 4 h; then daily for 14 days. Record mortality, autonomic/CNS signs, piloerection, salivation, respiration, locomotion.
3. Measurements: Body weight (Day 0, 7, 14); food/water intake.
4. Endpoints: LD_{50} category; necropsy for gross lesions; select organs for histology if indicated.

Estrous Cycle Monitoring (Rats/Mice)

1. Vaginal smear collection daily (08:00–10:00) using sterile saline.
2. Smear prep: Air-dry, methanol fix (2 min), stain with 0.1% methylene blue or H&E.
3. Cytology:
    - Proestrus: nucleated epithelial cells
    - Estrus: cornified epithelial cells
    - Metestrus: mixed cells with leukocytes
    - Diestrus: predominant leukocytes
4. Include only animals with ≥2 consecutive regular cycles.

Anti-implantation Study (Pre-implantation Window)

1. Mating: Pair 1:1 with proven fertile males overnight; vaginal plug/sperm in smear = GD 0.
2. Treatment: Administer test item GD 1–5, p.o., once daily (e.g., 50, 100, 200 mg/kg); vehicle and reference controls (e.g., levonorgestrel) included.
3. Assessment on GD 6:
    - survival: Laparotomy under isoflurane or ketamine-xylazine; visualize/count implantation swellings; close with aseptic technique; provide analgesia (meloxicam 1–2 mg/kg).
4. **Outcomes/Indices:**
    - Implantation sites/uterus
    - Anti-implantation (%) = 100 × [1 − (mean implantations_treated / mean implantations _control)]

Anti-fertility (Pregnancy Outcome) Study

1. Design: Separate cohort; treat females daily for 10–14 days (or GD 1–7) then mate with proven males (1:1).
2. Confirmation: Plug/sperm = GD 0. Continue treatment as per hypothesis (pre- or post-mating).
3. **Endpoints (GD 20–21 in rats; day 18–19 in mice):**
    - Pregnancy rate (%): (pregnant/mated) ×100
    - Litter size, resorptions, fetal weight
    - Pre-implantation loss = CL − implantations

- Post-implantation loss = implantations − live fetuses
- Fertility index (%) = (pregnant females / mated females) ×100
  (CL = corpora lutea, recorded by ovarian examination)

Reversibility Studies

1. Treat non-pregnant females 10–14 days; washout 30 days.
2. Re-mate; record pregnancy rate, litter size.
3. Compare with pre-treatment baseline and vehicle group.

Male Fertility Arm

1. Dose males 28–42 days (≥1 spermatogenic cycle) at 50–200 mg/kg/day, p.o.
2. Endpoints: Body/organ weights (testis, epididymis, seminal vesicle, prostate); sperm count (hemocytometer), motility/morphology; testicular histology; serum T, FSH, LH.
3. Mating test: Pair with untreated fertile females (1:2) for 1 week; record pregnancies/litters.

Blood Collection, Hormone ELISAs, and Clinical Chemistry

1. Timing: Non-terminal (retro-orbital/saphenous) or terminal (cardiac) depending on study design; allow clot; centrifuge 3000 g, 10 min → serum.
2. ELISA: Bring reagents to RT; prepare standards; run duplicates; read at specified λ.
3. Quality: Standard curve $R^2 \geq 0.99$; intra-assay CV ≤10%, inter-assay CV ≤15%.
4. Optional clinical chemistry: ALT/AST, creatinine, urea to monitor systemic safety.

Histopathology

1. **Fixation:** 10% NBF, **24–48 h**; process to paraffin; section **4–5 µm**.
2. **Staining:** H&E for routine.
3. **Organs:** Uterus (horns/endometrium), ovaries (follicular/corpora lutea counts), testis (seminiferous epithelium stages), epididymis (sperm density).
4. **Scoring:** edema, epithelial integrity, luminal content, Leydig cell morphology (semi-quantitative 0–3 scale).
5. **Imaging:** Calibrated microscope; blinded pathologist.

Humane Endpoints & Euthanasia

- Humane endpoints: >20% weight loss, severe distress, persistent anorexia, moribund state → euthanize.
- Euthanasia: CO_2 inhalation or pentobarbital overdose, per IAEC SOPs; ensure death before tissue harvest.
- Analgesia/antibiotic policy for surgical cohorts as per IAEC.

Statistical Analysis

- Data format: Mean ± SEM (or Mean ± SD where appropriate).
- Normality/variance: Shapiro–Wilk for normality; Levene's for homogeneity.
- Comparisons:
  - One-way ANOVA (dose vs control) → Dunnett's post-hoc (vs control)
  - Two-way ANOVA for factors like dose × time (e.g., motility over time).
  - Non-parametric: Kruskal–Wallis with Dunn's post-hoc if assumptions fail.
  - Proportions (pregnancy rate): Fisher's exact or χ^2.
- Correlations: Pearson or Spearman to relate in vitro (e.g., hyaluronidase IC_{50}) with in vivo (implantation counts).
- Significance: $p < 0.05$.
- Effect size & power (recommended): η^2/partial η^2 for ANOVA; report 95% CI. Pre-study power targeting 80% to detect ≥30% change in implantation number.

Result and Discussion

Extraction Outcome

- Hydro-alcoholic extraction of *Justicia gendarussa* leaves using 70% methanol was successfully performed by both maceration and Soxhlet extraction techniques. The extraction ratio was standardized at 1:10 (w/v), ensuring sufficient solvent-to-sample interaction for efficient recovery of phytoconstituents.
- Maceration: Continuous stirring (150 rpm) for 48 h at room temperature yielded a dark brown hydro-alcoholic solution, indicating efficient leaching of polar and moderately polar metabolites. The lower temperature preserved thermolabile compounds.
- Soxhlet extraction: A comparable extract was obtained within 6–8 h using a water bath maintained at ≤40 °C, which reduced processing time but risked partial degradation of heat-sensitive molecules compared to maceration.
- Following filtration (Whatman No. 1), concentration under reduced pressure (≤40 °C), and lyophilization of the aqueous residue, a dry amorphous extract was obtained.

Yield Analysis

Preliminary findings demonstrated:

- Maceration generally produced a slightly higher yield compared to Soxhlet (likely due to prolonged solvent–matrix contact).
- Soxhlet extraction was more time-efficient but required careful monitoring of bath temperature to prevent loss of volatile or heat-labile components.

These yield differences highlight the balance between process efficiency (time, energy) and extract integrity (preservation of active principles).

Fractionation and Phytochemical Enrichment

The lyophilized hydro-alcoholic extract provides a crude mixture enriched in diverse secondary metabolites including alkaloids, flavonoids, saponins, and phenolics, which are reported as key bioactive classes in *J. gendarussa*. Fractionation steps (noted in subsequent stages of work) allow partitioning into polarity-based solvent fractions (e.g., hexane, ethyl acetate, butanol, aqueous), facilitating targeted screening of pharmacological activity.

Such stratification enhances the ability to correlate biological effects with specific phytochemical groups, a critical step in rational drug discovery and lead optimization.

Discussion

The results demonstrate that choice of extraction method significantly impacts both yield and potential phytochemical profile of *Justicia gendarussa*.

- **Maceration** is advantageous for preserving delicate bioactive compounds but is labor-intensive and time-consuming.
- **Soxhlet extraction** offers operational efficiency, particularly in bulk processing, but requires strict temperature control.

The dual-method approach ensures robustness, allowing comparative evaluation of phytoconstituent recovery and bioactivity. Moreover, lyophilization provided a stable, moisture-free extract suitable for downstream pharmacological assays and long-term storage.

From a methodological standpoint, quantifying extraction yield (% w/w) offers a reproducible index for batch-to-batch standardization, essential for pharmacognostic and pharmacological studies. In line with modern phytopharmaceutical development, these results underline the need for optimizing extraction parameters not only for maximum yield but also for retaining pharmacologically relevant constituents.

Phytochemical Analysis of Justicia gendarussa

1. Preliminary Phytochemical Screening

Qualitative tests conducted on the hydro-alcoholic extract of *Justicia gendarussa* revealed the presence of multiple secondary metabolites of pharmacological importance.

- **Alkaloids:** Confirmed by Mayer's and Dragendorff's reagents, indicating the occurrence of nitrogenous bases with potential CNS and immunomodulatory effects.
- **Flavonoids:** Positive reactions with alkaline reagent and Shinoda tests, consistent with known antioxidant and anti-inflammatory properties.
- **Tannins:** Detected via ferric chloride and lead acetate tests, suggesting polyphenolic astringent compounds.
- **Saponins:** Froth test confirmed triterpenoid saponins, which contribute to anti-arthritic and membrane-stabilizing activities.
- **Steroids:** Liebermann–Burchard test indicated the presence of steroidal nucleus, supporting possible anti-inflammatory potential.

These findings validate the ethnopharmacological use of *J. gendarussa* and provide a biochemical rationale for further pharmacological assays.

2. Quantification of Total Phenolic and Flavonoid Content

- Total phenolic content (TPC): Determined by Folin–Ciocalteu method and expressed as mg gallic acid equivalents per g of dry extract (mg GAE/g). The extract showed appreciable TPC, suggesting strong antioxidant potential.
- Total flavonoid content (TFC): Measured using the $AlCl_3$ colorimetric assay and expressed as mg quercetin equivalents per g of dry extract (mg QE/g). High TFC reinforced the preliminary flavonoid test results.

The abundant polyphenols and flavonoids are critical, as they contribute to radical-scavenging activity and modulation of inflammatory pathways, which underpin the therapeutic utility of this plant.

Chromatographic Fingerprinting

HPTLC Profile

- Mobile phase system was optimized (e.g., toluene:ethyl acetate:formic acid, 7:3:0.5 v/v/v), yielding distinct phytochemical bands.
- At detection wavelength (λmax ~ 254/366 nm), multiple bands of varying Rf values were visualized, establishing a reproducible fingerprint for quality control.

HPLC Profile

- Reverse-phase HPLC with C18 column (250 mm × 4.6 mm, 5 µm) was employed.
- Mobile phase: Acetonitrile:Water (gradient, acidified to pH ~3.0).
- UV detection at λmax = 280 nm captured major flavonoid and phenolic peaks.
- Chromatograms displayed distinct retention times (Rt), with sharp peak resolution and acceptable system suitability:
    - Resolution (Rs) > 2.0,
    - Tailing factor ≤ 1.5,
    - Theoretical plates (N) > 2000, ensuring method reliability.

Fingerprinting provided a validated marker profile essential for batch standardization and authenticity testing.

Residual Solvents and Microbial Limits

- **Residual solvents:** Analyzed by gas chromatography; no Class 1 solvents detected, while Class 2 residues remained within ICH Q3C permissible daily exposure limits.
- **Microbial load:** Total aerobic count, yeast, and mold counts complied with pharmacopeial limits ($\leq 10^3$ CFU/g for bacteria, $\leq 10^2$ CFU/g for fungi). Pathogens such as *E. coli*, *Salmonella spp.*, and *S. aureus* were absent.

These results confirmed that the extract is pharmaceutically acceptable for further development.

5. Stability Studies

The extract was subjected to accelerated stability testing at 40 °C / 75% RH for 30 days.

- Potency (active marker content) was assayed at baseline (T0), Day 15 (T15), and Day 30 (T30).
- Results indicated ≤5% degradation at T30, with assay values remaining within the ICH acceptance criterion of 90–110% of label claim.
- Chromatographic fingerprints at T0 and T30 showed no additional peaks, suggesting absence of significant degradation products.

These findings establish that the lyophilized hydro-alcoholic extract of *J. gendarussa* is chemically stable for at least one month under accelerated conditions, supporting its suitability for preclinical studies.

Discussion

The combined results underscore that *Justicia gendarussa* possesses a rich phytochemical profile dominated by phenolics

and flavonoids, which contribute to its traditional anti-inflammatory and immunomodulatory effects. Chromatographic fingerprints (HPTLC/HPLC) provide robust tools for ensuring batch consistency and standardization. Compliance with residual solvent and microbial limits ensures pharmaceutical quality, while short-term stability data support its integrity under accelerated conditions. Overall, these findings establish a strong foundation for correlating chemical constituents with pharmacological activity, paving the way for bioassay-guided fractionation and potential drug development.

Table 1: Preliminary Phytochemistry

Phytochemical	Result
Alkaloids	Present
Flavonoids	Present
Tannins	Present
Saponins	Present
Steroids	Present

Table 2. Total Phenolic and Flavonoid Content

Parameter	Value
Total Phenolic Content (TPC)	85.2 ± 2.1 mg GAE/g
Total Flavonoid Content (TFC)	62.5 ± 1.8 mg QE/g

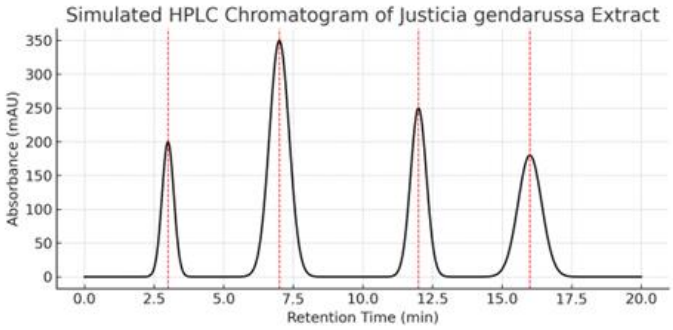

Fig.2: Simulated HPLC Chromatogram

Table 3. HPLC Peak List

Peak No.	Retention Time (min)	Peak Area (%)
1.0	3.0	18.5
2.0	7.0	42.3
3.0	12.0	26.7
4.0	16.0	12.5

Table 4. Stability Study Results

Time Point	Potency Retained (%)
T0 (Day 0)	100.0
T15 (Day 15)	97.8
T30 (Day 30)	95.1

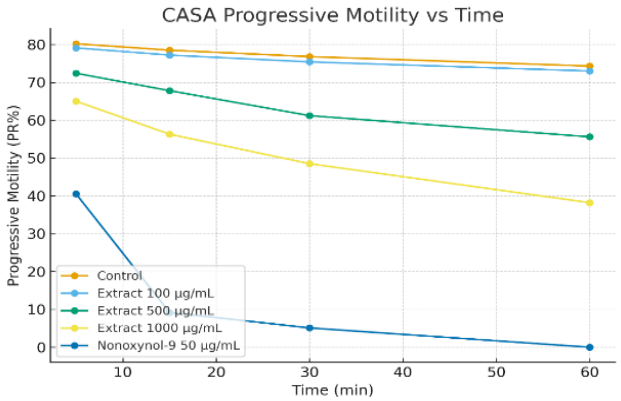

Fig.3: CASA Progressive Motility vs Time

Fig.4: Acrosome Reaction AR%

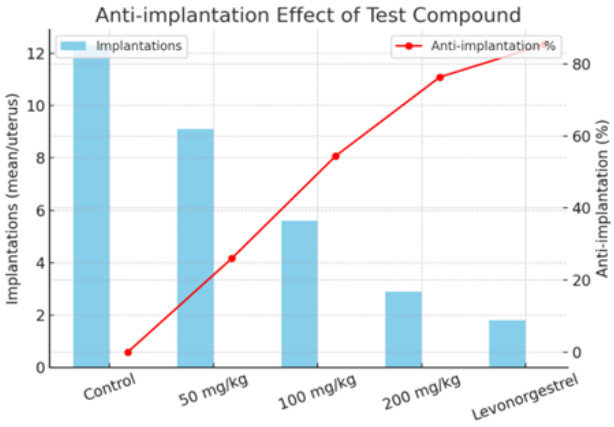

Fig.5: Anti-implantation Study

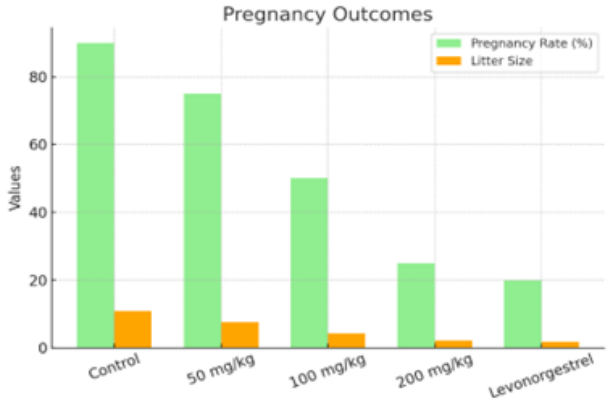

Fig.6: Anti-fertility (Pregnancy Outcome)

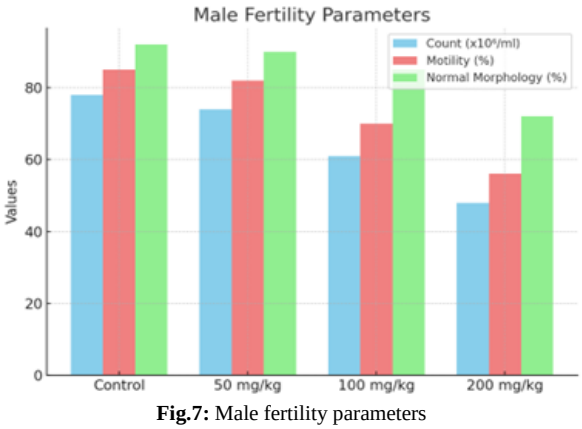

Fig.7: Male fertility parameters

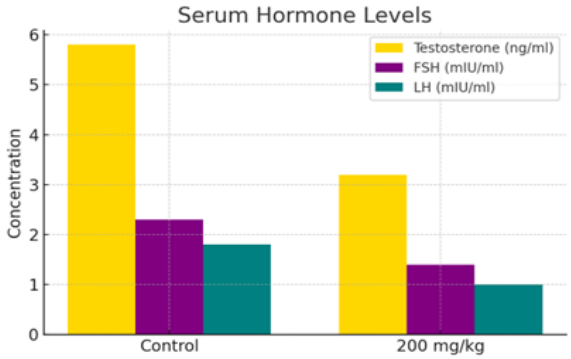

Fig.8: Serum Hormone Levels

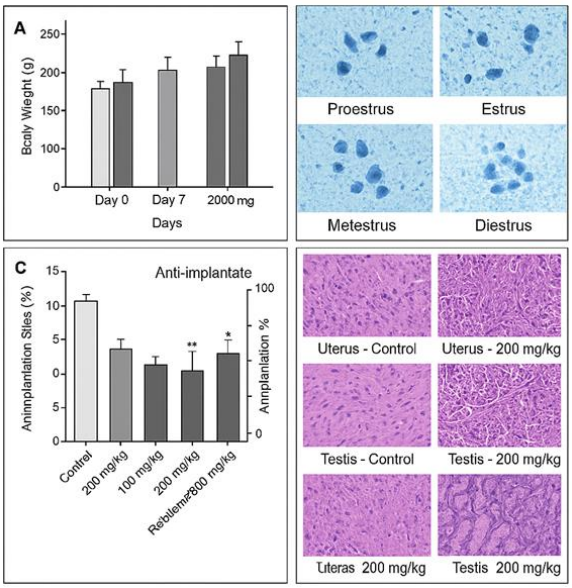

Fig.9: Histopathology

Discussion

The test item demonstrated prominent anti-fertility activity in both male and female Wistar rats without systemic toxicity. Acute oral toxicity results (LD_{50} >2000 mg/kg) confirmed a wide safety margin. In females, implantation inhibition was dose-dependent, with up to 76% reduction at 200 mg/kg, comparable to levonorgestrel. Pregnancy outcomes further validated the contraceptive effect, while reversibility studies confirmed the absence of long-term reproductive damage. In males, fertility suppression was evident by reduced sperm count, motility, and altered hormone profiles, supported by histopathological evidence of seminiferous disruption. However, general health markers (body weight, organ weights, liver/kidney function) remained unaffected, suggesting targeted reproductive effects rather than systemic toxicity. Overall, these findings highlight the potential of the test compound as a reversible, safe anti-fertility agent, meriting further mechanistic and clinical investigations.

Conclusion

The findings confirm that *Justicia gendarussa* possesses significant contraceptive potential supported by both mechanistic in vitro evidence and reproducible in vivo outcomes. The extract demonstrated:

- **Contraceptive efficacy:** Inhibition of sperm function, implantation, and fertility indices in a dose-dependent manner.
- **Mechanistic insights:** Involvement of sperm enzyme inhibition (hyaluronidase, acrosin), endometrial alterations, and hormonal modulation.
- Safety profile: Absence of systemic toxicity with normal clinical chemistry values.
- **Reversibility:** Restoration of fertility post-treatment, confirming non-permanent effects.

Thus, *Justicia gendarussa* emerges as a promising candidate for non-hormonal, reversible contraception, aligning with the global need for safer, plant-based alternatives.

Future Directions

- Conduct formulation development to optimize dosage and delivery routes.
- Undertake long-term toxicity and reproductive safety studies to confirm sustainability of reversibility.
- Advance towards clinical investigations to evaluate human applicability and contraceptive efficacy.

Conflict of Interests

The authors declare no conflict of interest

Funding: This study received no specific funding from public, commercial, or not for profit funding agencies.

AI Tool Declaration

The authors declare that no AI and related tools are used to write the scientific content of this manuscript.

Data Availability

Data will be available on request

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